

Invasion of dentinal tubules by root canal bacteria

ROBERT M. LOVE

Bacterial invasion of dentinal tubules and the clinical consequences have been recognized for over a century. However, while many components of the infected dentinal tubule microflora have been identified, it is likely that there are etiological agents involved in endodontic infections that have not yet been recognized. Bacterial invasion of coronal dentinal tubules occurs when the dentine is exposed to the oral environment and of radicular dentinal tubules subsequent to infection of the root canal system or as a consequence of periodontal disease. The content and architecture of a dentinal tubule can influence bacterial invasion, with tubule patency being important. This can account for regional variations in bacterial invasion and is particularly seen with dentinal sclerosis, where more advanced sclerotic changes in apical radicular tubules, especially in elderly individuals, limit bacterial invasion in this area. While several hundred bacterial species are known to inhabit the oral cavity, a relatively small and select group of bacteria are involved in invasion of dentinal tubules. Gram-positive organisms dominate the tubule microflora in both carious and non-carious dentine. The relatively high numbers of obligate anaerobes present, such as *Eubacterium* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Peptostreptococcus micros*, and *Veillonella* spp., suggests that the environment favors the growth of these bacteria. Gram-negative obligate anaerobic rods, e.g. *Porphyromonas* spp., are less frequently recovered; however, with time, fastidious obligately anaerobic bacteria become established as principal components of the microflora and can be found within the deep dentine layers. In the early stages of infection, Gram-positive bacteria dominate the microflora. The identification of adhesins that mediate these initial interactions of bacteria with dentine is important for understanding the development of tubule infection and in designing adhesion-blocking compounds. Recent evidence suggests that streptococci and enterococci may recognize components present within dentinal tubules, such as collagen type I, which stimulate bacterial adhesion and intra-tubular growth. Specific interactions of other oral bacteria with invading streptococci may then facilitate invasion of dentine by select bacteria. It is important therefore that the mechanisms of invasion and inter-bacterial adhesion are understood to assist development of novel control strategies.

Introduction

It is firmly established that bacteria are the prime etiological factor in the development and progression of dental caries and pulp and periapical disease. Over a century ago, Miller (1) first demonstrated bacterial invasion of dentinal tubules of both carious and non-carious dentine and reported that the tubule microflora consisted of cocci and rods. However, it was some time later before experimental evidence clearly established the fundamental role of bacteria in dental disease. Keyes (2) demonstrated that dental caries did not

develop in germ-free animals fed a range of diets, while Kakehashi et al. (3) showed that pulp and periapical disease occurred in surgically exposed rat molar pulp only when bacteria were present in the oral cavity. In gnotobiotic (germ-free) rats, exposed pulps remained healthy and initiated repair by way of dentine bridging of the exposure.

The pulp and dentine form a functional complex that is primarily involved in the production of dentine and in tooth sensibility and is protected from exogenous substances in the oral cavity by the overlying enamel or cementum. A breach in the protective layer may result

in disease; however, the pulpo-dentine complex possesses a number of mechanisms that react to bacterial invasion of dentinal tubules and exposed pulp in an attempt to eradicate them. The ability of the complex to perform this function should not be underestimated as the tissues are richly endowed with immunocompetent processes. However, in clinical terms, if the route of infection is not eradicated by these natural processes, or by operative procedures, then the burden of bacteria invading the complex overcomes the defenses and cause pulpitis, pulp necrosis, and infection of the pulp chamber and root canal system, and progresses to inflammatory periapical disease (Fig. 1).

Invasion of dentinal tubules by bacteria from supra- or sub-gingival plaque occurs whenever dentine is exposed in the oral cavity. This can be through carious lesions, restorative or periodontal procedures, tooth wear, enamel or dentine cracks, or dental trauma (4–7). Bacteria present within coronal dentinal tubules (Fig. 1A) may be responsible for pulpal disease (8) and subsequently take part in infection of the root canal system (Fig. 1B). However, bacteria that are associated with an infected root canal differ from those primarily associated with invasion of carious and non-carious dentine. Thus, although streptococci and *Actinomyces* are major components of dental plaque (9) and may initiate tubule and pulpal infection, obligately anaerobic bacteria are commonly present in large numbers in the infected root canal. Infection of the pulp space leads to bacterial invasion of radicular dentinal tubules (Fig. 1B,C) and the species involved is influenced by the development of the root canal flora. The presence of these bacteria may be responsible for continued root canal infection (10) and persistent apical periodontitis (Fig. 1E).

This article will review current knowledge of the microbiology of dentinal tubule infections, with an emphasis on the endodontic considerations of dentinal tubule invasion.

Structure of dentine and dentinal tubule

The relationship between enamel and cementum with dentine and the structure and composition of dentine matrix, and of the dentinal tubules, are key influences in the process of bacterial invasion of dentinal tubules. Dentine is porous, hard, mineralized connective tissue

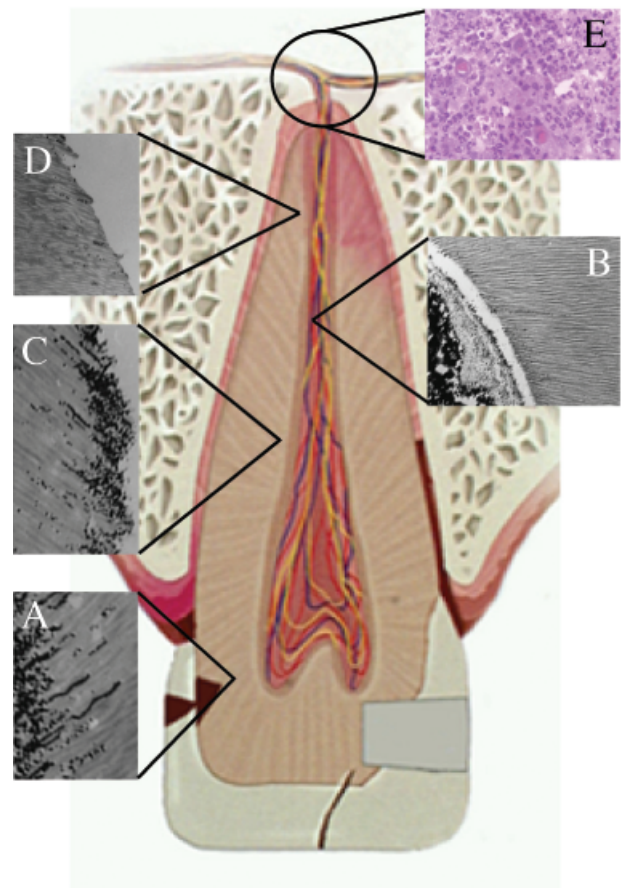


Fig. 1. Representative diagram showing potential routes of bacterial invasion of the pulpo-dentine complex and clinical consequences. (A) Bacterial invasion of dentinal tubules toward the pulp space as a result of a breach in the integrity of the enamel or cementum such as dental caries, enamel cracks/fractures, or restorative/periodontal procedures. (B) Bacterial biofilm in an infected root canal space with associated invasion of radicular dentinal tubules. (C) Moderate to heavy invasion of dentinal tubules of cervical and mid-root radicular dentine by root canal bacteria. (D) Low invasion of dentinal tubules of apical radicular dentine by root canal bacteria. (E) Histopathological section demonstrating bacteria-induced apical periodontitis subsequent to bacterial invasion of the root canal.

composed primarily of hydroxyapatite-coated collagen type I fibrils. Other collagen types (III, V, and VI) and non-collagenous proteins and proteoglycans are present as minor components (11). The matrix is formed by pulp odontoblast cells, which begin secreting collagen at the dentino-enamel junction and then retreat centripetally, trailing odontoblast processes around which the dentine matrix is elaborated and mineralized. This results in primary and secondary

dentine having a tubular nature with changes in the relative proportions of dentinal tubules within different areas of the dentine and with a characteristic S-shape course of the dentinal tubules. Consequently, tubules may contain odontoblast processes, nerve fibers, and unmineralized collagen fibrils (12).

The number of dentinal tubules per mm² varies from 15 000 at the dentino-enamel junction to 45 000 at the pulp (13). Deposition of intra-tubular (peritubular) dentine within the tubule results in narrowing of the tubule (11, 14). Deposition is more advanced in superficial older dentine compared with dentine closer to the pulp and this results in a tapered tubule, with the largest dimensions at the pulp (approximately 2.5 µm in diameter) and the smallest dimensions at the dentino-enamel or dentino-cemental junction (approximately 0.9 µm in diameter). Therefore, a tubule is normally larger in diameter than an average oral streptococcal cell (0.5–0.7 µm). Tertiary or reparative dentine, which is laid down as a consequence of noxious stimuli, does not have a regular tubular form.

Intra-tubular dentine is highly mineralized (approximately 95 vol% mineral phase) compared with the less mineralized collagen matrix (about 30 vol% mineral phase) of inter-tubular dentine (15), and becomes more mineralized with increasing age. This results in a decrease in size, and ultimately obliteration, of the dentinal tubules, with about a 40% decrease in the overall numbers between the ages of 20 and 80 years (14, 16, 17). The mean numbers of tubules at any given age within coronal, cervical, and mid-root dentine are similar (17). However, significantly fewer dentinal tubules are found in apical dentine (14, 17), suggesting that the formation of intra-tubular dentine occurs more rapidly in the apical region of the root and progresses toward the crown as a tooth matures (14).

Dentine is very porous because of the tubular structure, and once pathology, trauma, or restorative or periodontal procedures breach the integrity of the enamel or cementum, the underlying dentinal tubules provide diffusion channels to the pulp or periradicular tissues. Intact cementum is crucial to limiting bacterial invasion of radicular dentinal tubules from the pulpal surface of an infected root canal system. Bacterial penetration is enhanced when the overlying cementum is resorbed (10, 18, 19), a common occurrence in the presence of inflammatory periapical disease and after traumatic injuries that damage the periradicular tissues.

The degree of permeability varies between different areas of a tooth, the number of patent dentinal tubules present, and by tubule contents (5). The diameter of dentinal tubules influences the depth of bacterial invasion since this determines the rate of solute diffusion (20). Sclerotic or obliterated tubules will physically impede bacterial invasion and can result in regional differences in bacterial invasion of dentine, for example invasion of coronal and mid-root dentinal tubules occurs readily by *Streptococcus gordonii*, while the extent and depth of invasion are significantly less in apical tubules (19) (Fig. 1C,D). As discussed, this is because of the lower number of patent tubules in this region because of dentinal sclerosis, which is always more advanced in the apical region compared with coronal and mid-root dentine at any age (14) and lends weight to the notion that a chemomechanical root canal preparation with coronal flaring and minimal apical enlargement can effectively reduce the bulk of infected radicular dentine. Similarly, the presence of a dentinal smear layer, subsequent to tooth instrumentation such as filing a root canal, obliterates the superficial portion of tubules and prevents penetration of coronal or radicular dentinal tubules by streptococci *in vitro* (21, 22). This is confirmed by *in vivo* studies that demonstrated that bacterial invasion of dentinal tubules occurred more readily when the smear layer was removed, compared with smeared dentine where the degree of tubule invasion was low (23, 24).

The composition of dentinal tubule fluid in vital dentine is not fully known; however, it resembles serum, with proteins such as albumin and immunoglobulin G (IgG) being present (25). In addition, other blood proteins, such as fibrinogen, may be found in dentinal tubules after cavity preparation (25, 26). Similar molecules, derived from fluid originating from alveolar bone and periodontal ligament and saliva, are present in non-vital radicular and coronal dentinal tubules. It has been shown that albumin, fibrinogen, and IgG present within dentinal tubules decrease fluid flow through dentine *in vitro* (27, 28) and inhibit bacterial invasion of radicular dentinal tubules (29). Similarly, *in vitro* studies have demonstrated that fluid flow through dentine is reduced by bacterial invasion of dentinal tubules (21, 22).

It is likely that dentinal fluid components are involved in host defense, by directly interacting with bacteria and products, and by reducing the permeability of dentine. However, reduced fluid flow might promote disease

pathogenesis by allowing increased diffusion of destructive or toxic bacterial products toward the pulp and/or periradicular tissues (30).

Bacterial invasion of dentine

Several hundred bacterial species are recognized as components of the oral microflora; however, only relatively few species appear to be able to invade coronal dentine, infect the root canal system, and subsequently invade radicular dentinal tubules (31–33). This suggests that many species of oral bacteria do not have the necessary properties that allow invasion of tubules and their survival within the intra-tubular environment. The bacterial flora associated with invasion of radicular dentinal tubules is determined by the species of bacteria that invade coronal carious and non-carious dentine and infect the root canal; as such, this will be reviewed.

Carious coronal dentine

The cariogenic microflora present on the surface of fissure, smooth surface coronal, or root surface caries consists of mainly streptococci, lactobacilli, and *Actinomyces* spp. Members of the mutans group streptococci, in particular, *Streptococcus mutans* and *Streptococcus sobrinus*, are considered to be the primary etiological agents in the induction of coronal and of root caries (34–36). Samples of carious dentine from the outer surfaces of teeth contain *Streptococcus* spp., *Lactobacillus* spp., *Actinomyces* spp., and other Gram-positive rods (37). Samples from the pulpal side of carious dentine lesions of extracted teeth contain larger numbers of Gram-positive anaerobic rods of *Eubacterium*, *Propionibacterium*, and *Bifidobacterium* species, with *Actinomyces* and *Lactobacillus* being the most prevalent facultative bacteria isolated with streptococci comprising only a minor group of the total isolates (38). Thus, different regions of carious dentine may contain quite different proportions of bacterial components in their microflora.

Greater numbers of bacteria are recovered from superficial infected dentine compared with deeper dentine (39). Strict anaerobic sampling and cultivation methods reveal higher recoveries of bacteria, indicating that the environment of carious dentine promotes survival of obligately anaerobic bacteria. As a consequence, species of *Propionibacterium*, *Eubacterium*, and *Bifidobacterium* dominate the microflora of deep carious dentine, with *Actinomyces*, *Lactobacillus*, and

some streptococci being present, but rarely *S. mutans*. Gram-negative obligate anaerobes, e.g. *Fusobacterium*, are recovered in only very low numbers (Table 1). To identify and localize bacterial species within carious dentine, Ozaki et al. (40) detected, by immunohistochemical techniques, specific bacteria within dentine samples from fissure, smooth surface coronal, and root surface caries. They found that mutans group streptococci were the predominant bacteria within dentine from fissure and smooth surface coronal caries, with higher numbers in the shallow and middle layers of dentine compared with deep dentine. Other bacteria identified as being dominant members of the microflora of carious human dentine, such as *Lactobacillus* spp., *Eubacterium alactolyticum*, and *Fusobacterium nucleatum* (38, 39, 41), were frequently detected although their relative proportions were low (Table 1). The utilization of molecular methods to identify bacteria is a powerful technique and PCR has confirmed that *Streptococcus* spp. and *Lactobacillus* spp. are dominant species within deep carious dentinal lesions but that numerous novel taxa are present e.g. *Lactobacillus gasseri/johnsonii*, *Lactobacillus rhamnosus* (42). These observations show that the environment within superficial carious dentine favors the growth of facultative anaerobes that are associated with the carious process, e.g. mutans streptococci, while obligate anaerobic organisms dominate the microflora deep within the dentine.

In contrast to the microflora of fissure and smooth surface carious dentine, *Actinomyces naeslundii* (*viscosus*) is the major species associated with dentine invasion in root surface caries. *Actinomyces* species are found in shallow, middle, and deep dentine, with higher numbers of cells in deeper dentine. Mutans streptococci are frequently detected at all levels of carious root dentine, although they are mainly located in the shallow layer and do not make up a high proportion of the microflora. On the other hand, lactobacilli and Gram-negative organisms are found in low numbers, or not at all (40, 43, 44) (Table 2). Thus, the composition of the microflora associated with carious dentine differs quite considerably between coronal and root caries.

The microflora of carious and cavitated dentine of teeth with pulpitis is similar to that reported for intact carious dentine (45) (Table 1). Gram-positive organisms predominate, especially *Lactobacillus* spp. and streptococci. Gram-negative bacteria, e.g. *Prevotella intermedia*, are

Table 1. Bacterial species identified in carious coronal dentine (39, 49)

Bacterial genus or species	Isolation frequency in carious dentin		Bacterial genus or species	Isolation frequency in carious dentin	
	Superficial	Deep		Superficial	Deep
<i>Streptococcus</i>	High	Low-moderate	<i>Propionibacterium</i>	Moderate-high	High
<i>S. mutans</i>			<i>P. acnes</i>		
<i>S. sobrinus</i>			<i>P. avidum</i>		
<i>S. intermedius</i>			<i>P. lymphophilum</i>		
<i>S. morbillorum</i>			<i>P. propionicum</i>	Low	Moderate
<i>S. sanguinis</i>					
<i>Peptostreptococcus</i>	Low	Low	<i>Lactobacillus</i>	High	High
<i>P. anaerobius</i>			<i>L. casei</i>		
<i>P. parvulus</i>			<i>L. plantarum</i>		
<i>P. micros</i>			<i>L. minutus</i>		
<i>Actinomyces</i>	High	Moderate	<i>Fusobacterium nucleatum</i>	Low	Low
<i>A. israelii</i>					
<i>A. naeslundii</i>					
<i>A. odontolyticus</i>					
<i>Eubacterium</i>	High	High	<i>Peptococcus</i> spp.	Low	Low
<i>E. alactolyticum</i>					
<i>E. aerofaciens</i>					
<i>E. saburreum</i>					
<i>Veillonella</i> spp.	Moderate	Low	<i>Clostridium</i> spp.	Low	Low
<i>Bifidobacterium</i> spp.	High	High	<i>Porphyromonas</i> spp.	Low	Low
			<i>Prevotella</i> spp.	Low	Low

found in lower numbers in superficial dentine, but are more prevalent within dentine at the pulpal wall, while a positive correlation between the presence of *P. intermedia* and *Prevotella melaninogenica* and extensive pulp inflammation has been demonstrated (46).

Non-carious dentine

In vivo studies show that bacteria are able to penetrate into the tubules of non-carious coronal dentine

exposed to the oral environment. Invasion of tubules occurs readily and is evident within a week of exposure (24, 47). With time, the numbers of tubules infected and the depth of infection increase (47). The pattern of invasion is characterized by variable numbers of tubules penetrated and variable depths of penetration between different areas of dentine (Fig. 3) (4, 24). Inflammatory changes within the pulp are commonly observed and can be seen within a week of exposure (24). Other studies have demonstrated that microleakage of oral

Table 2. Bacterial species identified in carious root dentin (39, 49)

Bacterial species	Isolation frequency	Bacterial species	Isolation frequency
<i>Streptococcus</i>	Low-high	<i>Propionibacterium</i> spp.	Low-moderate
<i>S. sanguinis</i>		<i>P. acnes</i>	
<i>S. mitis</i>		<i>Lactobacillus</i>	Low
<i>S. mutans</i>		<i>L. plantarum</i>	
<i>S. sobrinus</i>		<i>L. casei</i>	
<i>Actinomyces</i>	High		
<i>A. naeslundii</i>		<i>Peptostreptococcus micros</i>	Low
<i>A. odontolyticus</i>		<i>F. nucleatum</i>	Low
<i>A. viscosus</i>		<i>P. endodontalis</i>	Low
Eubacterium spp.	Low-moderate	Veillonella spp.	Low
<i>E. alactolyticum</i>			

bacteria around restorations allows bacterial invasion of exposed dentinal tubules at the base of the cavity (8, 23) resulting in pulpal inflammation (23) or periapical disease (48). Likewise, microleakage through enamel cracks and fractures as a result of trauma may lead to bacterial invasion of the pulpo-dentine complex and act as a cause of endodontic disease (7). Hence, sealing of dentine from exogenous substances and bacteria in the oral cavity, in both vital and non-vital teeth, is a critical step in tooth restoration.

The composition of the microflora invading exposed non-carious dentine has not been fully elucidated but is dominated by Gram-positive cells (4, 8, 23, 24, 47) and probably resembles the composition of the biofilm infiltrating the tooth-restoration interface (49). This biofilm resembles mature plaque and is composed mainly of streptococci and *Actinomyces* spp. Anaerobic Gram-positive cocci, e.g. *Peptostreptococcus micros* and Gram-negative organisms, tend to be present in only low numbers (50, 51).

Microflora of the infected root canal

Bacteria may enter the root canal system directly via carious lesions or via pulp exposure following trauma. However, many infections of the pulp occur as a result of supra- or sub-gingival bacteria penetrating exposed

dentine, enamel-dentine cracks, and around restorations (5, 7, 52) and then invading dentinal tubules. Almost all bacteria recovered from the root canal systems of teeth belong to the oral microflora (32, 53, 54) and various factors such as nutritional supply, oxygen tension, and bacterial interactions influence the development of the root canal flora (55, 56).

Most of the oxygen-sensitive members of the root canal micro-flora are not readily cultivable without strict application of anaerobic methods (57) and this may explain why, in early studies, many teeth with apical periodontitis did not appear to harbor bacteria in the root canal. Utilizing strictly anaerobic sampling techniques, it has been shown that in addition to streptococci, lactobacilli, and *Actinomyces*, obligately anaerobic species of *Fusobacterium*, *Peptostreptococcus*, *Eubacterium*, *Propionibacterium*, *Veillonella*, *Wolinella*, *Prevotella*, and *Porphyromonas* dominate the established root canal microflora (32, 54, 58, 59) (Table 3). Other micro-organisms such as yeasts, e.g. *Candida* and *Saccharomyces* (60) and spirochetes, e.g. *Treponema* (61, 62) have also been recovered from an infected root canal. Recently, the use of molecular techniques to identify root canal bacteria has confirmed the presence of the dominant species (Table 3) as well as the presence of previously unidentified or unculturable bacteria such as *Dialister pneumosintes* (63, 64).

Table 3. Bacterial species commonly found in asymptomatic infected root canals (53–55)

Gram-positive cocci	Gram-positive rods	Gram-negative cocci	Gram-negative rods
<i>Streptococcus anginosus</i>	<i>Actinomyces israeli</i>	<i>Capnocytophaga ochracea</i>	<i>Fusobacterium nucleatum</i>
<i>S. sanguinis</i>	<i>A. naeslundii</i>	<i>C. sputigena</i>	<i>Prevotella intermedia</i>
<i>S. mitis</i>			<i>P. melaninogenica</i>
<i>S. mutans</i>	<i>Eubacterium alactolyticum</i>		<i>P. denticola</i>
	<i>E. lentum</i>	<i>Veillonella parvula</i>	<i>P. buccae</i>
<i>Enterococcus faecalis</i>	<i>E. nodatum</i>		<i>P. buccalis</i>
	<i>E. timidum</i>		<i>P. oralis</i>
<i>Peptostreptococcus micros</i>		<i>Campylobacter rectus</i>	
<i>P. anaerobius</i>	<i>Propionibacterium propionicum</i>	<i>C. curvus</i>	<i>Porphyromonas gingivalis</i>
	<i>P. granulosum</i>		<i>P. endodontalis</i>
			<i>Bacteroides gracilis</i>
	<i>Lactobacillus</i>		

Invasion of radicular dentinal tubules by root canal bacteria

Once bacteria gain access into the root canal system, they invade radicular dentinal tubules (Fig. 1C) and may be responsible for persistent root canal infection (10, 65). Shovelton (66) examined histologically 97 extracted, clinically non-vital teeth and found that 61 of the teeth showed bacterial penetration of the radicular dentinal tubules. The numbers of tubules containing bacteria were highly variable from tooth to tooth and among sections of an individual tooth. The depth of penetration by bacteria into the tubules was also found to be variable. It was noted that the presence of bacteria within the tubules was related to the clinical history of the tooth, such that chronic infections had more bacterial invasion and that tubule invasion did not occur immediately after the bacteria appeared in the root canal. These observations were similar to later histological studies on invasion of non-carious coronal dentine (4, 8, 23, 24, 47).

The microflora within radicular dentinal tubules of teeth with infected root canals (67) resembles that of deep layers of carious coronal dentine (38, 39) (Table 1). Lactobacilli, streptococci, and *Propionibacterium* spp. are predominant, with other bacteria such as Gram-positive anaerobic cocci, *Eubacterium* spp., and *Veillonella* spp. being present in low numbers. Obli-

gately anaerobic Gram-negative bacteria are recovered in very low numbers or not at all (38, 39, 67), but are known to be present in infected root canals as previously discussed. Additionally, yeasts have been reported to invade radicular dentinal tubules in teeth with root canals heavily colonized by yeasts (68). The inability to detect fastidious anaerobes within invaded coronal or radicular dentine may be because of difficulties in cultivating these bacteria. By utilizing specific antisera, Ozaki et al. (40) demonstrated that *Porphyromonas endodontalis* was present, albeit in low numbers, within dentinal tubules of carious dentine. Recently, Peters et al. (52) demonstrated that the flora recovered from mid-root radicular dentine of teeth with apical periodontitis of endodontic origin was similar to previous studies (67) while Gram-negative bacteria including *F. nucleatum*, *Porphyromonas gingivalis*, and *P. intermedia* were commonly recovered. These observations show that bacteria that are able to invade coronal dentinal tubules maintain the ability, under different environmental conditions in the root canal, to invade radicular dentine.

Bacterial invasion *in vitro*

In vitro studies have examined penetration of coronal or root dentine by a limited number of oral bacteria

that are associated with carious or non-carious dentine. Cells of *S. mutans*, *Streptococcus sanguinis*, and *A. naeslundii* have all been shown to penetrate dentine discs *in vitro* (21, 69, 70). Invasion of root dentinal tubules by pure cultures of streptococci or enterococci associated with root canal infections *in vivo*, or with dentinal caries, has been demonstrated histologically (10, 19, 22, 65, 71–74). In contrast, invasion of dentine by mono-cultures of Gram-negative anaerobic bacteria is less clear but invasion has not been generally recognized. Cells of *Bacteroides melaninogenicus* ss. *Melaninogenicus*, *P. intermedia*, or *P. gingivalis* (71, 72, 75) invaded radicular dentine. On the other hand, limited invasion by *P. intermedia* has been reported (76) while *P. endodontalis* and *P. gingivalis* both showed low-level penetration of dentinal tubules of bovine roots that had the cementum removed (77). Additionally, dentinal tubule invasion by *Candida albicans* has been reported (78, 79) and invasion may be species specific in this genus as it has been shown that *C. albicans* was the only one of five yeasts to invade dentine (80).

The ability of mixed cultures of bacteria, associated with coronal or root caries, to invade dentine was investigated by Nagaoka et al. (74). Their data suggested that invasion of *Lactobacillus casei* was enhanced when co-cultured with *S. sobrinus* or *A. naeslundii*. Similarly, it has been shown that dentinal tubule invasion by *P. gingivalis* was promoted when co-cultivated with *S. gordonii* (75). These experiments demonstrate that bacteria may compete for invasion of dentinal tubules, and also that they may co-operate in invasion. Both these interactions may be significant in determining the outcome of tubule infections.

Factors influencing dentinal tubule invasion

It is generally accepted that before bacteria can invade the human body, they must first colonize the host by undergoing a series of interactive events. Firstly, bacteria must adhere to the host tissue. Initially, this is by a loose physical association of the organism to the surface of a tissue that allows stronger and more permanent bonds to be established through binding of microbial cell surface adhesins to complementary host surface receptors. Once the microbial cells are bound, they must be able to utilize available nutrients, compete

or co-operate with other species in the immediate environment, and contend with host defense mechanisms before accumulation of micro-organisms can occur by cell division and growth. Once these processes occur at multiple sites the host becomes colonized. As such, bacterial invasion of dentinal tubules must follow these principles. Although tubule invasion has been recognized for over a hundred years, identification of the processes involved has only recently been elicited.

The central role of streptococcal interactions with deposited salivary proteins and glycoproteins on oral surfaces and other organisms in the development of dental plaque biofilms is well recognized (81–83). A large number of streptococcal protein adhesins have been identified that can interact with salivary molecules, including the antigen I/II family polypeptides (84); amylase-binding proteins (85); surface lectins (86, 87) fimbrial adhesins (88); EP-GP binding protein (89); and glucan-binding proteins GBP₇₄ (90) and GBP₅₉ (91). The possession of multiple adhesins favors colonization by a range of mechanisms. Inter-bacterial coaggregation is also an important aspect in plaque development; streptococci coadhere with other early colonizers, such as *Actinomyces* spp., and are also bound by later colonizers such as *P. gingivalis* and *Bacteroides forsythus* (92, 93). Later colonizers are often strict anaerobes and increase in plaque when a more anaerobic environment develops, which may be due, in part, to the actions of earlier colonizers. Despite extensive knowledge about adhesive interactions between bacteria and substrates in the oral cavity, the influence of bacterial adhesion and inter-bacterial binding in tubule invasion is relatively poorly understood.

Dentinal tubules contain appreciable amounts of unmineralized collagen (12). Collagen type I is recognized by oral streptococci, and it serves as an adhesion substrate when absorbed onto hydroxyapatite surfaces (94, 95). It has been demonstrated that strains of *S. mutans* are able to bind to unmineralized collagen and to particles of root dentine (96). The ability of oral streptococci to bind to collagen may facilitate bacterial adhesion to exposed dentine or cementum, and subsequently tubule invasion. The antigen I/II polypeptides, expressed on the surface of most indigenous species of oral streptococci (84), have been shown to be involved in mediating adhesion of streptococci to collagen (73), providing evidence for bacterial adhesion specificity as playing a major role in determining invasion of dentinal tubules. Utilizing isogenic mutants

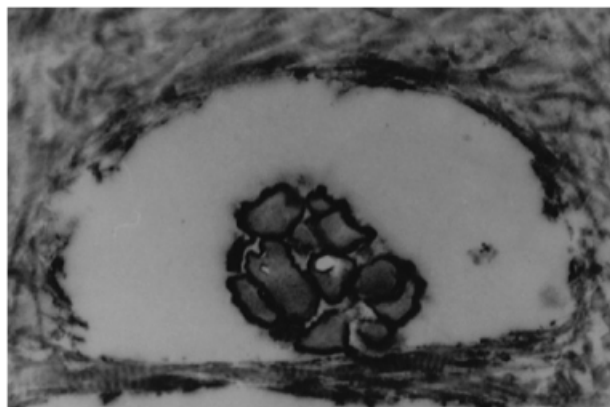


Fig. 2. Transmission electron micrograph demonstrating a colony of bacterial cells invading a radicular dentinal tubule. Note the close approximation of peripheral cells of the colony with the wall of the tubule indicative of cell attachment to tubule structure, an essential step in colonization.

of *S. gordonii* or *S. mutans* deficient in expression of antigen I/II polypeptide surface adhesins, it has been shown that these polypeptides mediate streptococcal binding to collagen and are necessary for bacterial invasion of dentine (73). Experiments suggest that bacterial cell recognition of type I collagen may facilitate bacterial adhesion to dentine (Fig. 2), as well as up-regulate production of antigen I/II polypeptide and induce a morphological growth response, manifested by long-chaining of streptococcal cells, facilitating tubule invasion (73). Additionally, a specific adherent interaction between *S. gordonii* and *P. gingivalis* cells mediated by the streptococcal antigen I/II polypeptides promoted tubule invasion by *P. gingivalis*, while the antigen I/II polypeptide SpaP of *S. mutans* did not have the same binding capacity or invasive affect on *P. gingivalis*, indicating a species-specific function of antigen I/II polypeptides (75). These observations demonstrate that tubule invasion and development of the inter-tubular bacterial flora follow that seen in the formation of plaque biofilms, that is, initial attachment and colonization by primary streptococcal colonizers, which then allow colonization by late colonizers. It is highly likely that other bacterial interactions between host proteins and other bacteria influence tubule invasion.

Limiting nutritional supply within a dentinal tubule may influence the depth of bacterial penetration. This is partly dependent upon the patency of the tubule as diffusion of substances into tubules from the oral cavity

or pulpal fluid in vital dentine or from the periradicular tissues in non-vital radicular dentine is proportional to tubule diameter. This may account for the higher numbers of cariogenic bacteria present within superficial dentine (49), where the presence of fermentable carbohydrates and oxygen from the oral cavity is likely to be higher than in deeper dentine. Also, the anaerobic environment and possible presence of tissue components, e.g. hemin, within coronal or radicular dentine close to the pulp is likely to favor growth and survival of organisms such as *P. intermedia* and *P. gingivalis* (28).

Microbiological aspects of persistent periapical disease

Persistent periapical pathology subsequent to endodontic treatment is because of intra-radicular infection (97), extraradicular infection (98), or other pathology e.g. a true cyst (99, 100). Intra-radicular infection is the most common cause of persistent pathology and the reasons for infection are numerous including lack of coronal seal, missed canals, insufficient debridement and disinfection of the root canal system, and therapy-resistant bacteria. Unlike primary root canal infections, which are typically mixed consisting of between two and eight bacterial species with obligate anaerobic bacteria dominating the microflora and streptococci making up a significant proportion of the facultative species, the root canal flora from failed cases is primarily Gram-positive facultative anaerobes and consists of 1–2 species per canal. The most frequently cultivable microorganisms include bacterial species from *Enterococcus*, *Streptococcus*, *Peptostreptococcus*, and *Actinomyces* (59, 101, 102), while molecular analysis of refractory endodontic cases has demonstrated the ability to detect the presence of unculturable bacteria such as *Dialister* (63, 103). Additionally, yeasts, notably *C. albicans*, have been isolated from cases of endodontic failure (97, 101, 102, 104). *E. faecalis*, which makes up a small percentage of the flora in primary root canal infection, is the bacterial species most frequently recovered in root-filled teeth, and often as a pure culture (102, 104).

For bacteria to be involved in the pathogenesis and maintenance of persistent apical periodontitis, they must be able to survive in the inhospitable environment of the obturated root canal where the nutrient supply is limited. Investigators have focused on *E. faecalis* in an

attempt to elicit mechanisms involved in pathogenesis. Studies have shown that *E. faecalis* is able to withstand a high alkaline environment such as the one generated by calcium hydroxide (10), and this appears to be related to a cell proton-pump that is necessary for its survival at high pH (105), and can form biofilms in calcium hydroxide-medicated canals (106). In addition, under starved conditions, it shows resistance to sodium hypochlorite (107), heat, hydrogen peroxide, acid, and ethanol (108). *E. faecalis* can also survive extended periods of starvation in water (109) and within water-filled dentinal tubules (65), and human serum (110), which likely reflects the nutritional supply within non-vital radicular dentinal tubules. The up-regulation of

stress-induced proteins has been shown to be important for cell survival in a stressed environment (109). It is highly likely that bacterial cells within dentinal tubules would be in a state of starvation and some of these mechanisms may come into play.

Additionally, *Enterococci* possess a number of virulence factors that permit adherence to host cells and extracellular matrix and facilitate tissue invasion. It is noteworthy that *E. faecalis* (110) and other bacterial species identified in refractory cases (59) possess the ability to invade dentinal tubules as mono-cultures in *in vitro* experiments (Fig. 3). It has been demonstrated that dentinal tubule invasion and adhesion to collagen by *S. mutans* or *S. gordonii* was inhibited by human serum, suggesting a protective mechanism of serum. In contrast, cells of *E. faecalis* maintained their ability to invade dentine and adhere to collagen in the presence of serum. It was suggested that following root canal therapy, this ability may allow residual *E. faecalis* cells in radicular dentinal tubules to re-colonize the obturated root canal and participate in chronic failure of endodontically treated teeth (110). Recently, using *E. faecalis* mutants deficient in serine protease and the collagen-binding protein (Ace), it was demonstrated that these molecules contribute to cell adhesion to radicular dentine (111) and as such are likely to be important in colonization and invasion of dentinal tubules. These data demonstrate that specific adherent interactions between oral bacteria may facilitate tubule invasion. The observations should stimulate more detailed investigations of other bacterial interactions and their role in determining the composition of the dentinal tubule and root canal microflora.

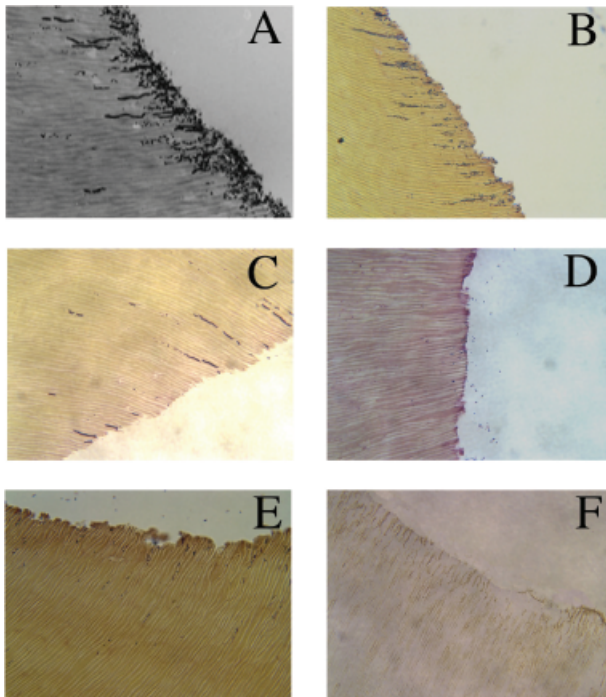


Fig. 3. Representative radicular dentine sections demonstrating dentinal tubule invasion by mono-cultures of some species commonly recovered from refractory endodontic cases (59). (A) *Streptococcus gordonii* (similar to *Streptococcus sanguinis*) showing characteristic heavy invasion (Brown and Brenn stain). (B) *Enterococcus faecalis* showing characteristic heavy invasion (Brown and Brenn stain). (C) *Streptococcus mitis* showing characteristic mild invasion (Brown and Brenn stain). (D) *Peptostreptococcus micros* showing characteristic low invasion (Brown and Brenn stain). (E) *Actinomyces naeslundii* showing characteristic low invasion (Brown and Brenn stain). (F) *Prevotella intermedia*, characteristic low invasion shown immuno-histochemically using antibodies raised to the species.

Conclusion

Invasion of radicular dentinal tubules by root canal bacteria is a multi-factorial event that a limited number of oral bacterial species have the necessary properties to participate in. Although the clinical endodontic techniques of chemomechanical root canal preparation (112, 113) and inter-appointment root canal medication (114) can manage infected radicular dentine, the complexity of the microbial interactions and bacterial resistance to treatment suggests that continued research into the pathologic events of invasion and development of the intra-tubular flora is warranted.

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